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(19)

(54) METHOD FOR DETERMINATION OF THE PRESENCE OF ANTIBIOTICS

(71) We, GIST-BROCADES N.V., a Dutch Body Corporate of 1 Wateringseweg, Delft, Holland, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention relates to a method for the rapid determination of the presence or absence of residues of antibiotic, for example penicillin, in a liquid (for example milk or liquid derived from meat), and to a test vessel to be used in this method.

Methods for the determination of antibiotic residues, more particularly penicillin residues, in milk and similar liquids have been known for a long time, and several standard methods are accepted officially for the purpose in some countries. Examples thereof are the so-called TTC-method (involving 2,3,5-triphenyltetrazolium chloride) such as the method of Neal *et al.*, J. Dairy Science *38* (1955), 629—633, and a method using *Bacillus stearothermophilus* or *B. calidolactis* of Galesloot *et al.*, Neth. Milk & Dairy J. *16* (1962), 89—95, based on a method of Vincent *et al.*, Proc. Soc. exp. Biol. Med. *162* (1944), 55, and the method of Jacobs *et al.*, Tijdschr. v. Diergeneesk. *79* (1972): 9 548—550. The method of Galesloot *et al* is carried out by placing paper discs, soaked in the milk to be tested, on agar cultures of *Bacillus stearothermophilus* or *B. calidolactis* on petri dishes and incubating at 55°C. during 2½ hours. Formation of inhibition zones is an indication of the presence of penicillin (or other substances inhibiting these bacilli) in the milk.

The method of Galesloot *et al* has a sensitivity of about 0.0025 IU (international units) of penicillin per ml., but this method is not suitable to be carried out by unskilled persons, because of the necessity to use fresh cultures of *B. stearothermophilus* or *B. calidolactis*. Furthermore, the reliability of this test is relatively low when carried out by less skilled persons.

In order to increase the reliability of the Galesloot method, H. Mol, Neth. Milk & Dairy J. *23* (1969), 153—162, makes use of spores of *Bacillus stearothermophilus var. calidolactis* prepared in petri dishes. Before use, a nutrient disc, obtained by soaking a piece of filter paper in an aqueous solution of 20% peptone and 20% of glucose followed by drying, is soaked in the sample of milk and placed on the agar. The petri dish is incubated for 6 hours at 63°C., and the presence of penicillin may be recognised by the presence of an inhibition zone.

Although in the method of Mol reliability is increased, the method still has several disadvantages. In the first place, it is difficult to recognise the inhibition zone, especially for unskilled persons. Furthermore, the volume of sample is insufficiently reproducible.

To be a suitable rapid test, the following requirements should be fulfilled:

(i) it should be rapid,
(ii) it should show a high sensitivity for a wide range of antibiotics used in practice,
(iii) the vessels containing test material should be storable for a reasonably long time (several months or longer),
(iv) the test should be cheap,
(v) the test should give reliable results, even when carried out by unskilled people, e.g. when the test is adapted to show the presence of a predetermined concentration of antibiotic, it should give sufficiently reliable positive or negative results.

Although the method of Mol fulfills requirements (i) to (iv) to a certain extent, requirement (v) is not fulfilled and qualified people are needed to carry out the method to obtain reliable results.

Taking the several ideas mentioned above into consideration, a method has been developed by us for the rapid determination of antibiotics in liquids which fulfills all five above-mentioned requirements, and thus is easily carried out even by unskilled persons.

According to the invention, a method is pro-

vided for the rapid determination of the presence or absence of residues of antibiotic, for example penicillin, in a liquid (for example milk or liquid derived from meat), which comprises introducing spores of a microorganism sensitive to the antibiotic to be determined, into a liquid agar medium containing insufficient nutrients to permit germination of the live spores, the spore culture thus obtained being allowed to solidify in upright transparent or translucent test vessels having an internal cross-sectional dimension of 3 to 20 mm., preferably 6—14 mm., placing on the agar surface nutrients required for growth of the spores of the microorganism, followed, if desired after a pre-incubation period, by adding a predetermined amount of sample liquid to be tested to the test vessel, the height of agar medium and sample liquid together preferably being 3—30 mm., more preferably 5—10 mm., and incubating at or near optimal temperature the contents of the test vessel for a predetermined time so that the extent of growth or inhibition of growth of the microorganism, into the agar medium, observed visually, indicates absence or presence of antibiotic.

It is to be understood that the term "antibiotic" used in this specification and accompanying claims includes chemotherapeutics, such as trimetroprim, sulfadoxin and furazolidone, to which the microorganisms used are sensitive.

When the method is standardised, the test can be carried out easily with reliable results, even by unskilled persons, and in some cases even a rough quantitative estimate of the amount of antibiotic present in the sample can be made, or the presence of a concentration exceeding the sensitivity may be detected. The solidification of the spore-containing agar medium when the test vessel is upright, combined with standardised amounts of sample and medium, has the advantage that the size of the surface area of the agar medium is well defined, so that, when introducing the nutrient compounds reliable incubation results are obtained, which may be semi-quantitative.

An indicator may be added to the agar medium containing the spores or to the nutrients required for growth of the spores of the microorganism. If an indicator is included in the agar medium, a better distribution in the medium is obtained before the test is carried out. Furthermore, the sensitivity of the test is higher, and for liquids other than milk the colour is less contaminated by colour impurities originating from the sample. The indicator may be an acid-base indicator such as bromocresol purple or phenol red, or a redox indicator such as 2,3,5-triphenyl-tetrazolium chloride. For the determination of penicillin, the use of bromocresol purple is preferred.

The pH of the medium is important for the optimal growth rate of the test microorganism as well as for the activity of the antibiotics to be tested, and thus for the sensitivity of the microorganism to the antibiotics. For *Bacillus stearothermophilus var. calidolactis*, for example, the pH of the medium should be about 7 in the beginning of the test period in order to obtain an optimal growth rate. By using a suitable indicator such as bromocresol purple, a very short test period may be achieved.

Although the test period will be somewhat longer, the sensitivity of the test to certain antibiotics, especially aminoglucoside antibiotics such as streptomycin, dihydrostreptomycin, kanamycin and neomycin, may be increased by starting the test with a medium having a pH of about 8. A suitable indicator in this case is, for example, phenol red. The sensitivity to the other antibiotics, however, is not influenced disadvantageously when the pH of the medium during the test decreases in such a manner that each antibiotic at its suitable pH inhibits the growth of the microorganism. The pH range between the start and the finish of the test may, if desired, be extended by using mixed indicators.

The sensitivity of the test to certain antibiotics may be adapted by using spores or combinations of spores which are particularly sensitive to the antibiotic to be determined.

For the purpose of the invention, spores may be used from all bacteria which can form spores and are sufficiently sensitive to the antibiotic(s) to be tested. A suitable spectrum of sensitivities to different antibiotics may be obtained by using spores of one species or strain, or by using a mixture of spores of different organisms. Suitable spores are those of spore producers of the genus *Bacillus* with suitable sensitivity to the antibiotics involved, such as *Bacillus calidolactis* (Hussong *et al.*, J. Bact. 15 (1928) 179—188), *Bacillus subtilis*, *Bacillus stearothermophilus* (Bergey's Manual of Determinative Bacteriology, 7th Ed., (1957) 613—693), the thermophilic *bacilli* described by Galesloot *et al.*, Neth. Milk & Dairy J. 13 (1959) 155—179, *Bacillus stearothermophilus var. calidolactis* (Mol, Neth. Milk & Dairy J. 23 (1969) 153—162) and *Bacillus calidolactis* strain C 953 of the Netherlands Institute of Dairy Research at Ede, The Netherlands (Galesloot *et al.*, Neth. Milk & Dairy J. 16 (1962) 89—95). Preferably, spores are used of *Bacillus stearothermophilis var. calidolactis*, which is deposited with the Laboratory for Microbiology at Delft, The Netherlands, and assigned the number L.M.D. 74.1. This microorganism has a high sensitivity to penicillin-G and penicillin-V, and a very high growth rate, and has the additional advantage that its optimal growth temperature is relatively high, whereat other microorganisms normally do not grow, so that the chance of infection is reduced. Spores of this microorganism are not only sensitive to penicillin-G

and penicillin-V and other natural penicillins, but are also sensitive to several other antibiotics, such as semi-synthetic penicillins, e.g. nafcillin and cloxacillin; cephalosporins; aminoglucoside antibiotics, e.g. streptomycin, dihydrostreptomycin, neomycin and kanamycin; tetracyclines, e.g. chlorotetracycline; chloramphenicol; macrolide antibiotics, e.g. oleandomycin and erythromycin; polypeptide antibiotics; and several chemotherapeutics, e.g. trimetropine, sulfadoxin and furazolidone, so that the method according to the invention is also useful to indicate the presence of these antibiotics and chemotherapeutics, if necessary, after adaption of the pH.

For use in accordance with the invention, the agar culture medium preferably contains 10^5 to 10^8 spores of microorganism per ml. of agar medium, more particularly 10^6 to 10^7 spores per ml. of medium. A suitable manner of preparing the spores of the microorganism is cultivation of the microorganism in surface culture on agar or in a submerged liquid culture, e.g. in shaken flasks or in a fermenter, as described by Yao *et al.*, Appl. Microbiol 15 (1967) 455 to produce spores. The spores are incorporated in the agar medium in a manner such that the spores stay alive but are prevented from germination. Physiological salt solution and/or an indicator may be added to the agar, but it should not contain sufficient nutrients to permit germination of the spores of the microorganism.

It is an additional advantage of the method according to the invention that the influence of inhibitors, e.g. products produced by leucocytes which are normally present in milk and other liquids to be tested and which are not antibiotics, is decreased to a considerable extent, which is normally not the case in previously known methods. This decrease of influence of these inhibitors is due to the fact that they do not, or do not to a substantial extent, diffuse into the agar inside the test vessels, e.g. test tubes. It is evident that this fact increases the reliability of the method of the invention, which has the further advantage of being a handy test since pre-treatment of the milk to inactivate those inhibitors is normally not necessary, even when samples of milk with a slightly altered appearance are presented.

The agar medium containing the spores is allowed to solidify in upright test vessels. Those vessels preferably have predetermined sizes in order that the test results are sufficiently reliable. The test according to the invention is even quantitative to some extent as, when the test is carried out under certain predetermined circumstances, the antibiotic diffuses into the agar, forming a gradient of decreasing concentrations in the agar. In the region where the concentration of the antibiotic is below a certain level, inhibition of growth will not occur, so that the medium is influenced by the growth of the microorganism, and the indicator when present changes its colour. The region where the concentration of the antibiotic is above this level, inhibition of growth occurs and the indicator will not change its colour. The height of the agar medium in the test vessel, e.g. test tube, where the colour of the indicator changes defines the sensitivity of the test. The sensitivity may be ascertained in the vessels according to the invention by standardising the height of the solidified agar in the vessel. This feature may be used advantageously to develop a test in which the height of the spore-containing agar is selected in such a manner that the indicator changes its colour when the antibiotic is present in a concentration below a certain value, but does not change its colour when the concentration of the antibiotic is above this value. The cross-sectional dimension of the test vessels is preferably 6—14 mm., and the height is such that the vessels may contain an amount of medium and sample corresponding to a height of preferably 3—30 mm. When the test is arranged to be a quantitative test, i.e. a test in which some quantitative results may be read from the height of the inhibition zone, this height is preferably 20—30 mm. When the test is arranged to be a test from which one can read whether a certain concentration of antibiotic is present or not, the height is preferably 5—15 mm.

When filled with a predetermined amount of the liquid agar medium, the contents are allowed to solidify when the test vessels are in upright position, so that a horizontal agar surface is obtained. The test vessels containing the solidified agar medium containing the spores may be closed, in which condition they may be stored for at least several months, if necessary in a refrigerator, without the spores losing their viability.

When carrying out the test for the presence of an antibiotic, the required nutrient compounds, which may contain an indicator, are first placed on the surface of the spore-containing agar medium in the test vessel. Although a predetermined amount of the nutrient compounds in liquid form may be placed on the surface of the agar medium, it is preferred to apply the nutrients in dry form, preferably in the form of filter paper discs or tablets, containing the required nutrient compounds in a dried state, so that the nutrients have a better storage stability. The discs or tablets should be smaller than the cross-sectional area of the vessel so as to prevent air entrapment when the disc or tablet is laid on the medium; air entrapment would influence the diffusion pattern in the agar. Nutrients to be used should contain at least an assimilable carbon source and nitrogen source, preferably in the form of glucose and peptone, depending on the strain applied. The nutrient discs or tablets may con-

tain an indicator and/or buffer.

After placing the nutrient compounds on the surface of the agar medium, a predetermined amount of the liquid to be tested, generally milk or liquid derived from meat, is added to the contents of the test vessel. This may be done directly after placing the nutrients on the surface of the agar medium, or after a pre-incubation period. When the nutrients are in a dry form, in the pre-incubation method, some water or physiological salt solution may also be added. The latter procedure has the advantage that, for example, when a batch of milk arrives in a factory where it is used for certain purposes, e.g. for distribution as milk for human consumption, for butter and cheese making or for yoghurt fermentation, which may only be done after the absence of penicillin (or other antibiotic) residues has been established, the period during which the batch should wait before being further used until the results of the test are known may be shorter. By applying a pre-incubation period, the incubation in the presence of the sample to be tested may be shorter than when a pre-incubation period is not applied. The pre-incubation period may be, depending on the circumstances, up to about one hour.

It is also possible to make a more economical use of the time during which the liquid to be tested, generally milk, is transported to the factory by starting the test during the transportation, using thermoblocs to keep the temperature constant. By the term "thermoblocs" is meant blocks of a substance having a high specific heat, to be placed around the test vessels according to the invention in order to minimise changes in temperature. Also, a combination of the above-indicated measures may be applied.

The incubation period (including the pre-incubation period) is dependent on the circumstances e.g. the spores used, and the nutrient and temperatures applied. When using spores of *Bacillus stearothermophilus var. calidolactis*, suitable incubation temperatures are about 55° to 70°C., preferably 60° to 65°C., and incubation periods, within which reliable results may be obtained, are relatively short and vary from about 1.5 to 4 hours, preferably from 2 to 3 hours.

Although the test has been developed especially for application to milk, the test may also be used for other materials such as liquid derived from meat, e.g. meat liquid squeezed from a sample of meat, or drip liquid from deep-frozen and defrosted meat. For that purpose, the nutrients must be adapted to neutralise disturbing factors. This may be done by the addition of suitable chemicals, e.g. pH buffers, to the test. Disturbing factors such as lactoferine and seroferine may be neutralised by the addition of ferrous sulphate. As a pre-screening for the residues of antibiotics in slaughter animals, the test can be applied to urine as antibiotics accumulate therein. For testing undiluted urine, the pH should be adapted to the test by use of suitable pH buffers in the tablet. Urine samples may also be tested without using additives, in this case it is necessary, however, to dilute the samples about 10 times with water.

In order to determine whether an antibiotic present in a sample is penicillin or another antibiotic, it is possible to carry out the test with two test vessels. To one of the vessels a nutrient tablet or an additional tablet or disc is added containing penicillinase, while to the other vessel a nutrient tablet is added without penicillinase addition. When penicillinase is present, the non-penicillinase resistant penicillins will be decomposed during the test. Another specific penicillin inactivator instead of penicillinase, such as cysteine, could also be used.

According to another feature, the invention provides a test vessel for the rapid determination of residues of antibiotic, for example penicillin, in a liquid (for example milk or liquid derived from meat), comprising spores of a microorganism sensitive to the antibiotic to be tested in an agar medium containing insufficient nutrients to permit germination of the live spores, the surface of the agar medium being substantially perpendicular to an upstanding side wall of the test vessel, the test vessel having an internal cross-sectional dimension of 3 to 20 mm. and being transparent or translucent. The test vessel may have the preferred dimensions in width and height heretofore particularly mentioned.

It will be appreciated that combinations of a certain number of test vessels, e.g. a block of translucent material provided with a number of holes shaped to form test vessels according to the invention, are also within the scope of the present invention. In another embodiment, the vessels, which may be in the form of test tubes or ampoules containing the spore suspension, may be combined to make a set by means of a suitable rack or basket.

As mentioned above, the test vessels containing the agar medium may be stored during a prolonged period depending on maintenance of suitable storage conditions, especially temperatures.

The pH of the agar medium in the test vessel can be affected during storage by the ambient air, probably due to the presence of carbon dioxide in the air. Therefore, the test vessels, such as ampoules, are preferably sealed air-tight during storage.

Sometimes, the agar tends to loosen from the wall of the vessel. This tendency may be decreased by adding to the agar a sticking agent increasing cohesion of the agar to the wall of the vessel, which agent should not act as a nutrient or an inhibitor for the spores. Examples of suitable sticking agents are sodium carboxymethylcellulose and sorbitan

esters known as Tween (trade mark).

According to still another feature, the invention provides a set for the rapid determination of residues of antibiotic, for example penicillin, in a liquid (for example milk) or liquid derived from meat), comprising one or more test vessels as indicated above and a corresponding number of discs or tablets containing the required nutrients in dried form as hereinbefore mentioned, and optionally means for placing a disc or tablet on the surface of the agar medium in each test vessel and applying a predetermined amount of sample liquid onto the agar surface. Such a set may be wrapped in a suitable packaging material such as Styropore (a foamed polystyrene) plastic material ("Styropore" is a registered Trade Mark).

The test vessels according to the invention may also contain within them the nutrient discs or tablets. As the discs or tablets withdraw moisture from the agar medium, they are coated with a layer which prevents moisture transport from the medium into the tablets or discs at ordinary or storage temperatures; the layer should, however, allow transport of the nutrients into the agar medium under the test conditions. An example of a suitable coating for the discs or tablets is a coating of wax having a melting temperature of about 35° to 55°C., preferably 40° to 45°C.

The set may also contain other useful attributes, for example, a check picture from which a rough estimation of the penicillin (or other antibiotic) content may be read from the height of the inhibition zone. It may further comprise means for applying a predetermined amount of sample to the surface of the agar medium. Preferably, one blank sample without penicillin or other antibiotic, e.g. plain water, may be included to control the good working of the set.

As hereinbefore indicated, the method according to the invention may be used for the determination of antibiotic residues, for example penicillin residues, in milk. However, residues of antibiotics in other liquids may also be determined by means of the method according to the invention, for example, residues in the liquid which is obtained by squeezing meat, drip liquid, liquid derived from organs, e.g. kidneys, or from food- and feedstuffs, and other liquids such as blood serum and urine. If necessary, buffers may be added to control the pH of the liquid. Different buffers may be employed depending on the source from which the liquid is obtained.

It will be appreciated that other microorganisms may be used to detect other antibiotics, if used in practice. This, of course, may involve adaptation of the medium, the nutrients and the incubation conditions.

The invention is illustrated by the following Examples.

EXAMPLE I.

Preparation of test tubes.

A culture of *Bacillus stearothermophilus var. calidolactis* L.M.D. 74.1 is inoculated on a medium consisting of:

Bacto nutrient agar, Difco code 0001 (Difco is a registered Trade Mark)	15 g.
Bacto agar, Difco code 0140	5 g.
dextrose	0.5 g.
$MnSO_4 . H_2O$	30 mg
distilled water to	1000 ml

sterilised for 20 minutes at 120°C. After inoculation, the medium is incubated at 60°C. for at least 48 hours until a good sporulation is observed. The spores are collected, washed with distilled water and stored at 4°C.

The amount of viable spores is detected by testing on a medium consisting of:

Bacto agar, Difco code 0140	20 g.
Bacto Tryptone, Difco code 0123	8.5 g.
Phytone Peptone, BBL code 11905	1.5 g.
dextrose	5 g.
distilled water to	1000 ml

sterilised for 20 minutes at 120°C. After inoculation, the medium is incubated for 48 hours at 60°C., after which the colonies are counted.

Distilled water is added to, or water is removed from, the spore suspension until the suspension contains about 10^8 viable spores per ml. One percent of the above-mentioned spore suspension containing 10^8 germs per ml. is added to the following solution:

Bacto agar, Difco code 0140	12 g.
sodium chloride	9 g.
distilled water to	1000 ml

sterilised for 20 minutes at 120°C. The medium is liquefied by heating, and cooled to 60°C.

Sterile test tubes having cross-sectional dimensions of about 9 mm. are each filled with 0.5 ml of the thus obtained medium. The contents of the test tubes are allowed to solidify, the tubes being held in the upright position. The tubes are stored at a temperature of 4°C.

Preparation of nutrient discs.

The following media are prepared:

(a)	Bromocresol purple (0.1 g.) dissolved in 9.2 ml. of 0.02N NaOH is diluted with distilled water to make 25 ml.	
(b)	dextrose	50 g.
	distilled water	50 ml.
(c)	Bacto Tryptone, Difco code 0123	34 g.
	Phytone Peptone, BBL 11905	6 g.
	distilled water	100 ml.

The solutions (a) and (b) are sterilised by passage through a Seitz filter ("Seitz" is a registered Trade Mark); medium (c) is sterilised at 110°C. for 30 minutes: it remains a suspension. Five parts of solution (a), two parts of solution (b) and three parts of suspension (c) are mixed together, and 0.04 ml. of the solution obtained is contacted with filter paper discs having a cross-sectional dimension of about 8 mm., and the discs are then dried.

EXAMPLE II.

Carrying out a test.

Amounts of penicillin-G are added to fresh cow's milk to obtain concentrations of 0.1, 0.03, 0.01, 0.003 and 0.001 IU per ml., while one sample of cow's milk without penicillin-G is run as a blank test. Six test tubes, as prepared according to the method of Example I, are each provided with a nutrient disc and 0.2 ml. of each of the samples are added to the test tubes. Immediately, the test tubes are placed in a water bath at 65°C. Observations are made after 3 and 4 hours. The results are:

Blank sample: the agar colours yellow;
The samples with less than 0.01 IU per ml: the agar is coloured yellow;
The samples with 0.01 IU per ml. and higher: the medium is coloured violet.

EXAMPLE III.

Sensitivity of the test compared with sensitivities of known test.

In the following Table, the sensitivity of the contents of the test tubes prepared according to Example I is compared with sensitivities of known tests for the determination of residues of antibiotics: a kidney test prescribed in The Netherlands and described by Schothorst and Peelen-Knol in Tijdschr. v. Diergeneesk. *95* (1970) 438—445; and a German method developed by the German Bundesgesundheits-Amt, cf. Levetzow, Bundesgesundheitsblatt 14 (1971) 30—42 and Bartels *et al.*, Die Fleischwirtschaft 52 (1972) 479—482. The test according to the invention is carried out in two ways: in the first place the test is carried out with the antibiotics indicated below dissolved in saline, and in the second place with milk contaminated with the indicated antibiotics. The first way gives an impression as to what may be expected when the test is carried out as a meat test.

It is clear from the Table, in which the sensitivities are indicated in μg./ml., that the test according to the invention is more sensitive than the kidney test for most of the antibiotics indicated. This is also true for the comparison with the German test where it will be appreciated that the test according to the invention is much more sensitive to penicillin.

TABLE

	Antibiotic	Test of Invention		Kidney test S. lutea 40 ml. per plate	Modified kidney test; S. lutea 20 ml. per plate		German method B. subtilis	
		Upper limit in phys. salt sol.	Lower and upper limit in milk		pH 6	pH 8	pH 6	pH 8
1	penicillin Na-salt	0.004	0.003–0.005	0.04	0.02	0.02	0.01	0.01
2	kanamycin	10	20–30	ca. 200	ca. 200	16	3	0.9
3	neomycin	2	2–8	ca. 600	ca. 400	1	10	0.1
4	streptomycin	10	18–22	80–100	40	1	1	0.2
5	erythromycin	0.8	1.5–3	1–2	0.8–1	0.05	20	0.8
6	oleandomycin	5	5–10	8–10	7	0.05	20	0.2
7	spiramycin		>10	ca. 150	ca. 100	0.4	>200	2
8	oxytetracycline	0.1	0.2–0.5	2–4	2	ca. 10	0.5	8 (?)
9	chlorotetracycline		0.15–0.4	ca. 0.8	0.6	1	0.08	0.4
10	tetracycline-HCl	0.05	0.15–0.4	2–4	2	>10	0.5	8
11	rifamycin		0.03–0.04	0.03	0.01	0.02	0.02	0.04
12	zinc bacitracin	0.1	0.2–0.4	1	0.6	4	ca. 300	ca. 500
13	chloramphenicol	8	5–7.5	5–8	4	6	5	8
14	nafcillin	0.04	0.01–0.02	0.2	0.1	0.1	4	>10
15	furazolidone	5	?–8	ca. 100	ca. 60	>100	1	2
16	sulfamezathine		>1000	ca. 2000	ca. 1000	ca. 700	ca. 700	ca. 200

TABLE (Continued)

	Antibiotic	Test of Invention		Kidney test S. lutea 40 ml. per plate	Modified kidney test; S. lutea 20 ml. per plate		German method B. subtilis	
		Upper limit in phys. salt sol.	Lower and upper limit in milk		pH 6	pH 8	pH 6	pH 8
17	1)		0.6–0.8	50 S; 12 T	20 S; 4T	8 S; 1.2 T	5 S; 1 T	4 S; 0.8 T
18	spectinomycin	4	1–?	ca. 1000	ca. 600	30	70	30
19	lincomycin	0.6	?–0.5	ca. 2	1	0.1	500	5
20	cloxacillin		0.003–0.004	0.4	0.2	0.4	0.2	0.4

1) 200 mg. sulfadoxin + 40 mg. trimetroprim

WHAT WE CLAIM IS:—

1. A method for the rapid determination of the presence or absence of residues of antibiotic in a liquid which comprises introducing spores of a microorganism sensitive to the antibiotic to be determined into a liquid agar medium containing insufficient nutrients to permit germination of the live spores, the spore culture thus obtained being allowed to solidify in upright transparent or translucent test vessels having an internal cross-sectional dimension of 3 to 20 mm., placing on the agar surface nutrients required for growth of the spores of the microorganism, followed, if desired after a pre-incubation period, by adding a predetermined amount of sample liquid to be tested to the test vessel, and incubating at or near optimal temperature the contents of the test vessel for a predetermined time so that the extent of growth or inhibition of growth of the microorganism into the agar medium, observed visually, indicates absence or presence of antibiotic.

2. Method according to claim 1 in which the liquid is milk.

3. Method according to claim 1 or 2 in which the antibiotic is penicillin.

4. Method according to claim 1, 2 or 3 in which the cross-sectional dimension of the test vessel is 6 to 14 mm.

5. Method according to claim 1, 2, 3 or 4 in which the height of the sample to be tested and the agar medium together is 3 to 30 mm.

6. Method according to claim 1, 2, 3 or 4 in which the height of the sample to be tested and the agar medium together is 5 to 10 mm.

7. Method according to any one of claims 1 to 6 in which the spore culture in the agar medium comprises an indicator.

8. Method according to any one of claims 1 to 6 in which an indicator is incorporated in the nutrients required for growth of the spores of the microorganism.

9. Method according to claim 7 or 8 in which the indicator is an acid-base indicator.

10. Method according to claim 9 in which

the indicator is bromocresol purple or phenol red.

11. Method according to claim 7 or 8 in which the indicator is a redox indicator.

12. Method according to claim 11 in which the redox indicator is 2,3,5-triphenyl-tetrazolium chloride.

13. Method according to any one of claims 1 to 12 in which the pH of the agar medium is about 8 at the beginning of the test period.

14. Method according to any one of claims 1 to 13 in which spores are used of a species of the genus *Bacillus*.

15. Method according to claim 14 in which spores are used of *Bacillus calidolactis, Bacillus subtilis, Bacillus stearothermophilus* or *Bacillus stearothermophilus var calidolactis.*

16. Method according to claim 15 in which spores are used of *Bacillus stearothermophilus var. calidolactis* (L.M.D. 74.1).

17. Method according to claim 16 in which the pH of the agar medium is about 7 at the beginning of the test period.

18. Method according to any one of claims 1 to 13 in which a mixture of spores of different organisms is used.

19. Method according to any one of claims 1 to 18 in which the spores culture contains 10^5 to 10^8 spores of microorganism per ml. of agar medium.

20. Method according to claim 19 in which the spore culture contains 10^6 to 10^7 spores of microorganism per ml. of agar medium.

21. Method according to any one of claims 1 to 20 in which the spores of the microorganism are prepared by cultivation of the microorganism in surface culture on agar or in a submerged liquid culture to produce spores of the microorganism.

22. Method according to any one of claims 1 to 21 in which the nutrients placed on the agar surface are applied in a dry form.

23. Method according to claim 22 in which the nutrients are in the form of filter paper discs or tablets.

24. Method according to claim 22 in which the nutrients in the form of filter paper discs or tablets are coated with a layer preventing moisture transport from the agar medium into the discs or tablets at ordinary or storage temperature but allowing nutrients transport into the agar medium under the test conditions.

25. Method according to claim 24 in which the coating layer consists of wax having a melting temperature of 35° to 55°C.

26. Method according to claim 24 in which the coating layer consists of a wax having a melting temperature of 40° to 45°C.

27. Method according to claim 14 or 15 in which the incubation temperature is 55° to 70°C.

28. Method according to claim 14 or 15 in which the incubation temperature is 60° to 65°C.

29. Method according to claim 14 or 15 in which the incubation period is $1\frac{1}{2}$ to 4 hours.

30. Method according to claim 14 or 15 in which the incubation period is 2 to 3 hours.

31. Method according to claim 1 substantially as hereinbefore described.

32. Test method for the rapid determination of residues of antibiotic in a liquid, comprising spores of a microorganism sensitive to the antibiotic to be tested in an agar medium containing insufficient nutrients to permit germination of the live spores, the surface of the agar medium being substantially perpendicular to an upstanding side wall of the test vessel, the test vessel having an internal cross-sectional dimension of 3 to 20 mm. and being transparent or translucent.

33. Test vessel according to claim 32 in which the cross-sectional dimension is 6 to 14 mm.

34. Test vessel according to claim 32 or 33 in which an indicator is present in the spore culture in the test vessel.

35. Test vessel according to claim 34 in which the indicator is an acid-base indicator.

36. Test vessel according to claim 35 in which the indicator is bromocresol purple or phenol red.

37. Test vessel according to claim 34 in which the indicator is a redox indicator.

38. Test vessel according to claim 37 in which the redox indicator is 2,3,5 - triphenyltetrazolium chloride.

39. Test vessel according to claim 32 in which the pH of the agar medium is about 8.

40. Test vessel according to any one of claims 32 to 39 in which the spore culture is a spore culture of a species of the genus *Bacillus.*

41. Test vessel according to claim 40 in which the spore culture is a spore culture of *Bacillus calidolactis, Bacillus subtilis, Bacillus stearothermophilus* or *Bacillus stearothermophilus var. calidolactis.*

42. Test vessel according to claim 41 in which the spore culture is a spore culture of *Bacillus stearothermophilus var. calidolactis* (L.M.D. 74.1).

43. Test vessel according to claim 42 in which the pH of the agar medium is about 7.

44. Test vessel according to any one of claims 32 to 39 in which the spore culture is a spore culture of a mixture of spores of different organisms.

45. Test vessel according to any one of claims 32 to 44 in which the spore culture contains 10^5 to 10^8 spores of microorganism per ml. of agar medium.

46. Test vessel according to claim 45 in which the spore culture contains 10^6 to 10^7 spores per ml. of agar medium.

47. Test vessel according to any one of claims 32 to 46 in which the vessel contains, in addition to the spore culture in the agar medium, nutrients in the form of filter paper

discs or tablets coated with a layer preventing moisture transport from the agar medium into the discs or tablets at ordinary or storage temperatures, but allowing transport of nutrients into the agar medium under the test conditions.

48. Test vessel according to claim 47 in which the coating layer on the discs or tablets consists of wax having a melting temperature of 35° to 55°C.

49. Test vessel according to claim 47 in which the coating layer on the discs or tablets consists of wax having a melting temperature of 40° to 45°C.

50. A number of test vessels comprising a block of translucent material provided with a number of holes shaped therein to form test vessels according to any one of claims 32 to 49.

51. Set for the rapid determination of residues of antibiotic in a liquid comprising one or more test vessels as claimed in any one of claims 32 to 46 or 50, and further comprising a corresponding number of discs or tablets containing the required nutrients in dry form.

52. Test vessel according to claim 43, or set comprising one or more test vessels as claimed in claim 47, 48 or 49 and further comprising means for applying a predetermined amount of sample liquid to the surface of the agar medium.

53. Test vessel according to claim 32 substantially as hereinbefore described.

54. Set according to claim 51 substantially as hereinbefore described.

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